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The Biological Impact of the Human Master Regulator p53 Can Be Altered by Mutations That Change the Spectrum and Expression of Its Target Genes†

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Human tumor suppressor p53 is a sequence-specific master regulatory transcription factor that targets response elements (REs) in many genes. *p53* missense mutations in the DNA-binding domain are often cancer associated. As shown with systems based on the yeast *Saccharomyces cerevisiae*, p53 mutants can alter the spectra and intensities of transactivation from individual REs. We address directly in human cells the relationship between changes in the p53 master regulatory network and biological outcomes. Expression of integrated, tightly regulated DNA-binding domain *p53* mutants resulted in many patterns of apoptosis and survival following UV or ionizing radiation, or spontaneously. These patterns reflected changes in the spectra and activities of target genes, as demonstrated for *P21*, *MDM2*, *BAX*, and *MSH2*. Thus, as originally proposed for "master genes of diversity," p53 mutations in human cells can differentially influence target gene transactivation, resulting in a variety of biological consequences which, in turn, might be expected to influence tumor development and therapeutic efficacy.

Master regulators provide coordinated expression of vast networks of genes that often have broad biological consequences. These transcription proteins mediate transactivation through sequence-related response elements (REs). Although many factors determine levels of expression and spectra of genes expressed, individual sequences play an important role. While many studies have addressed the impact of mutational inactivation or changes in levels of these regulators, little is known about functional mutations that might alter the networks controlled by master regulators or their biological impacts, except for the human tumor suppressor p53.

As a transcription factor, p53 mediates many cellular responses to genotoxic insults and hypoxia (40, 56, 57). Through coordination of over 50 genes, activated p53 is central to a variety of biological functions including cell cycling, apoptosis, differentiation, cellular senescence (28, 30, 39), angiogenesis (6, 10), and the removal of DNA damage (16, 37, 49, 60, 66). Analysis of p53-regulated global gene expression reveals differences in strength, kinetics, and specificity that depend on the levels of p53, its posttranslational modifications, its degradation, nature of stress, cell type, and other as yet unidentified parameters (2, 46, 63, 65). It is likely that subsets of genes can be chosen from the complex spectrum of potentially inducible genes to mediate a specific p53 response in a given physiological situation (58, 65).

Inactivation of p53 regulation appears as a common step in tumor development (18, 22) since *p53* mutations are associated with nearly half of human cancers. Among the *p53* mutations associated with ~20,000 tumors (38, 52) the most frequent changes are missense mutations in the DNA-binding domain (DBD) of the protein. These can lead to nuclear accumulation of mutant p53 protein and loss of its normal functions, such as transcriptional activation of target genes (5, 38). However, the functional consequences of only ~10% of the nearly 1,100 different amino acid changes have been examined in any detail in mammalian cells.

While *p53* mutations can result in a null phenotype for transactivation of p53 target genes, many change its impact on transcription. Some can result in phenotypes different from simple null mutants, due to altered interactions with proteins that associate with p53, and may lead to transactivation genes that are normally not regulated by p53 (11, 29, 48, 51) (often referred to as "gain of function"). Most mutants that retain function exhibit changes in transactivation of p53 target genes (26, 43, 44). This is clear from studies with model systems based on the yeast *Saccharomyces cerevisiae* that address the consequences of missense mutations on the potential for p53 to transactivate from REs derived from many human target genes (23, 42). Surprisingly, mutations in the DBD can have a variety of effects, including general reduction in transactivation, altered spectrum in terms of which REs can support transactivation, changes in level of transactivation, and even transcription from sequences that are poorly recognized by wild-type p53. Collectively, these mutant p53s can be considered as altered rather than null for transactivation of target genes. In this regard, we had developed a highly regulatable (i.e., "rheostatable") p53 system in yeast to address transactivation capacity of wild-type and p53 mutants at over 15 human p53 target REs. Single amino acid changes in the p53 sequence-specific transcription factor could result in a variety of distinct transactivation spectra. We proposed that various alterations in p53 might result in a diverse set of biological responses that, in turn, could influence tumor development. In

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a general sense, p53 might represent a class of sequence-specific transcription factors that, in addition to being master regulators, are also "master genes of diversity" (42).

Based on the results in yeast, we have addressed directly in human cells tumor-associated p53 mutants that are functionally altered in transactivation in order to evaluate the relationship between changes in the p53 master regulatory network and biological outcomes. The p53 mutants were chosen from classes we initially identified in yeast as supertransactivating ("supertrans"), change of spectrum, and inactive. We found that among a sample of only eight p53 mutants, there were five unique patterns of biological consequences, just in terms of spontaneous and damage-induced apoptosis as well as clonal survival in response to UV or ionizing radiation. The reasons for the changes in phenotypes can be attributed to altered patterns of gene activities as exemplified by transcription, occupancy, and protein expression of a sample of endogenous p53 target genes—*P21*, *BAX*, *MSH2*, and *MDM2*—following expression of the various p53s at biologically meaningful levels.

MATERIALS AND METHODS

Cell lines and transfection. Human osteosarcoma SaOS2 (p53 null) and U2OS (p53 wild type) cell lines obtained from ATCC were maintained in Dulbecco's modified Eagle medium or McCoy's 5A with 10% FBS and antibiotics. For transient transfections, cells were seeded at 2×10^5 cells/six-well plate. After 24 h, cells were transfected with 2 μg of purified endotoxin-free plasmid expression vectors in the presence of nonliposomal transfection reagent FuGENE 6 (Roche) according to the manufacturer's protocol.

Plasmids. Plasmids pC53-SN3 (27) containing human p53 cDNA under control of the cytomegalovirus promoter and the empty vector pCMV-Neo-Bam were provided by Bert Vogelstein. Constructs carrying mutant p53s were constructed by replacing the SgraI-StuI *p53* portion with the same fragment of pLS89 derivatives containing the *p53* mutations (23, 24). *p53* constructs were confirmed by sequencing. Tet-Off cell lines were created with the pBI-EGFP plasmid (BD Biosciences Clontech).

SaOS2 cell lines with inducible p53 expression. p53 tetracycline-inducible SaOS2 cell lines were established with a Tet-Off gene expression system (BD Biosciences Clontech). Tetracycline-responsive p53 expression constructs were made by cloning restriction fragments of wild-type (wt) and mutant p53 cDNAs into the tetracycline (Tet)-responsive reporter plasmid pBI-EGFP using the pCMV-p53 constructs described above. Constructs were confirmed by sequencing. The plasmids containing the p53s were cotransfected into SaOS2 Tet-Off cells (Clontech), which express the tetracycline-controlled transactivator (17) along with *p*-puromycin in the presence of transfection reagent FuGENE 6. Single colonies were obtained by limiting dilution with 400 μg/ml G418 and 250 μg/ml puromycin in the presence of 1 μg/ml doxycycline (dox) for 2 to 3 weeks. To obtain the stable cell lines (at least five for each construct), potential clones were first selected with puromycin. After two rounds of selection, putative cell lines were identified. Clones that had low background green fluorescent protein and homogeneous green fluorescent protein induction after removal of dox were selected and analyzed for p53 expression by Western blot analysis. p53 expression was kept "off" by 2 μg/ml dox. To induce the p53s, cells were washed 3× with phosphate-buffered saline (PBS) and placed in medium lacking dox.

Growth suppression. Growth suppression was determined as reduction in colony formation. Cells (10^5) were plated on a 35-mm plate, and 24 h later were transfected with 1.5 μg of p53 expression plasmid or pCMV-Neo empty vector. Forty-eight hours later, the cells were trypsinized and 10^2 cells/well were plated on a six-well plate. After 3-week G418 selection (0.5 mg/ml), colonies with more than 50 cells were determined. Colonies were methanol fixed, Giemsa stained, and counted.

Impact of UV and γ irradiation on cell survival. For UV and ionizing radiation (γ), SaOS2 Tet-Off-inducible cell lines were grown in six-well plates in the presence of dox. Six hours prior to treatment, the cells were washed 3× in PBS. For UV treatment, cells were irradiated in a thin layer of PBS using a germicidal lamp at 1 J m^2/s. For γ treatment, cells were irradiated at 1.56 Gy/min from a Shepherd cesium irradiator in the culture medium at room temperature. A clonogenic assay was used to determine cell survival. After irradiation, cells were trypsinized, resuspended in fresh growth medium with no dox, and reseeded at 10^2 cells/well in a six-well plate. Clonal survival was determined as described for the growth suppression assay.

Apoptosis and cell cycle evaluation. Apoptosis was detected by morphology, fluorescence-activated cell sorter (FACS), and annexin V assays. For morphology, cells were stained with 5 mg/ml propidium iodide and 50 mg/ml acridine orange as described by Qutob and Ng (41). The percent apoptosis (>300 cells counted) was determined with a fluorescence microscope. For cell cycle and apoptosis evaluation by FACS analysis, adherent and detached cells were combined and fixed overnight with 75% ethanol in PBS at 4°C. After rinsing 2× with PBS, cells were incubated for 30 min with 1 ml of PBS containing 1 mg boiled RNase at 37°C. Cells were stained in 1 ml of PBS containing 1 mg of propidium iodide, and $\sim 10^4$ cells were analyzed in a flow cytometer (FACSort; Becton Dickinson). Annexin analysis was done with the V-PE/7-AAD apoptosis detection kit (BD Pharmingen) according to protocol on $\sim 10^4$ stained cells using a flow cytometer, where single positive staining for phycoerythrin (PE) corresponds to early apoptotic events, whereas double staining for PE and 7-aminoactinomycin (7-AAD) represents both late-apoptosis and necrotic cells. Absence of staining for both fluorochromes corresponds to live cells.

Western blot analysis. Equal amounts of whole-cell extracts were separated on BisTris NuPAGE (4 to 12%) and transferred to polyvinylidene difluoride membranes (Invitrogen). The blots were probed with primary antibodies and bands were detected using horseradish peroxide-conjugated secondary antibodies (Santa Cruz) and the enhanced chemiluminescence detection system (Amersham). Specific monoclonal antibodies were as follows: p53 (pAb1801 and DO-1), p-p53 (Ser15-R), p-p53 (Ser392), hMdm2 (C-18), Bax (N-20), p21 (C-19), Gadd45 (C-20), Msh2 (N-20), and actin (C-11).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were as described by Kaeser and Iggo (25) using 1 μg mouse monoclonal anti-p53 antibody DO7 (BD Pharmingen). Amplification and PCR conditions are described in the supplemental material.

TaqMan real-time PCR. Relative gene expression levels and differences among transient and stable transfected p53 SaOS2 cells were validated by TaqMan real-time PCR (20). The approach is detailed in the Materials and Methods in the supplemental material. Reactions were carried out in 96-well plates using the ABI PRISM 770 sequence detection system platform (PE Applied Biosystems) according to the manufacturer's recommendations.

Luciferase assay. For luciferase assays in the p53 null SaOS-2 cell line, 10^5 cells were seeded in 24-well plates 24 h before transfection. Cells at 80% of confluence were subjected to transfection with FuGENE 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) following the manufacturer's instructions. The cells were cotransfected with 100 ng of pGL3 reporter plasmid, 25 ng of pCMV-p53wt or p53 mutant expression vector, and 75 ng of pRL-SV40. In all cases the total amount of plasmid DNA per well was adjusted to an equal level by adding the empty vector pCMV-Neo. Cell cultures were harvested 48 h after transfection, and the luciferase activities were assessed using the dual-luciferase assay system (Promega) according to the manufacturer's protocol. The firefly luciferase activity was normalized with *Renilla* luciferase activity for each sample. For all cell lines, experiments were performed at least twice in triplicate.

RESULTS

Functionally altered p53 mutants create a variety of phenotypic changes in human cancer cell lines. The p53 network of genes contributes to the biological outcomes of apoptosis, cell proliferation, and DNA repair. Previously, using an isogenic yeast-based system, we examined the in vivo transactivation capacity of wild-type human p53 and 25 partial-function mutants, mainly located in the p53 DNA-binding domain (42). The p53s were expressed in yeast from a "rheostatable" promoter, and the transactivation capacities toward 15 promoter REs upstream of a reporter gene were measured. Quantitative transactivation revealed that single amino acid changes in p53 can lead to considerable diversity in the spectrum of responses from the different REs relative to the wild type in the following areas: (i) decrease/loss of function, (ii) subtle changes, (iii) altered specificity, and (iv) "supertransactivation." We therefore determined the biological impact of p53 mutants that, according to results in yeast, might differentially change the

ability to influence genes within the network. Representatives from mutant classes we had identified in yeast as supertrans (S121F, T123P, H178Y, and N288K), change of spectrum (T125R and G279R), and inactive (G245S and G279E), as well as wild-type p53, were examined for their impact on several biological responses. Each of them, except T123P, has been identified in human cancers (see Table S1 in the supplemental material). Presented below and in Fig. 1 to 5, as well as in the figures in the supplemental material, are the results for both transient and stable regulatable transgenes; the results are summarized in Tables 1 and 2.

Transfected p53 mutants result in many different phenotypes. Initially, we determined the ability of the various p53 mutants to influence growth suppression and apoptosis when transfected into a cancer cell line, SaOS2, which does not express p53. The pCMV-Neo plasmids carrying the wild type or a specific *p53* mutant were transfected using FuGENE 6 transfection reagent (see Materials and Methods). At 24 h posttransfection, p53 protein levels were comparable (Fig. 1A). To evaluate the ability of mutant p53s to affect overall clonogenic survival in human cells, colony forming ability was determined by selection for the p53 expression vector neomycin (G418) marker 2 weeks after transfection (see Materials and Methods). As shown in Fig. 1B and C, the mutants exhibited dramatic differences in the ability to reduce colony formation and induction of apoptosis 24 h after transfection.

Transfection with wt p53 induced apoptosis in nearly 30% of the cells and led to an 80% reduction in clonal survival. The G245S and G279E mutants which are inactive for transactivation and, in the case of G245S, unable to bind p53 target REs (8, 36, 61), had little impact on these biological end points. Cells expressing the supertrans S121F and N288K mutants were clearly more effective at inducing growth inhibition and apoptosis than the other altered alleles, resulting in ~60% apoptotic cells versus 30% with wt p53 (Fig. 1C). The other two alleles that were supertrans in yeast, T123P and H178Y, were much closer to the wild type in their effects, as was G279R. The T125R mutant appeared to be intermediate between the wt and null mutants in its effects. Mutations in the same domain, and even amino acid changes at the same position (G279) in the second helix domain, can have different biological impacts. As summarized in Table 1, six of the eight mutants clearly retained p53-related growth suppression and apoptotic functions when ectopically expressed in SaOS2 cells. The retention of biological activity for most of these mutants has also been observed for transfection into other cell lines that have endogenous wt p53 activity (data not shown). Based on the results with the transfected p53s, there are several distinct patterns of biological responses for the eight mutants examined.

Differences in biological consequences of stably expressing p53 mutants with altered transactivation activities. To better characterize the biological consequences of the various p53 mutants and to address effects on additional end points, SaOS2 cell lines were created containing p53 mutants that are tightly regulated. The p53s were regulated by the Tet-Off (BD Biosciences Clontech) tetracycline-controlled system. In the absence of the tetracycline derivative dox, the transactivator binds to the tetracycline response element in the minimal cytomegalovirus promoter allowing it to drive expression of the p53 cDNA. Transcription can be turned off in a dox-dose-dependent manner (17).

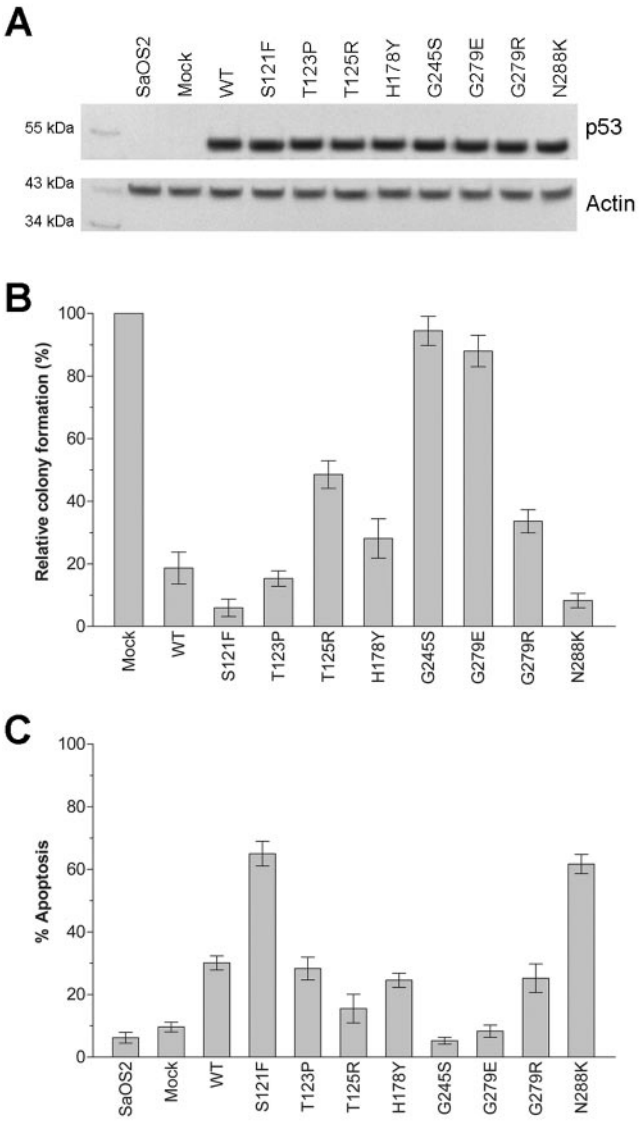


FIG. 1. Transient transfection of p53 mutants changes biological outcomes (phenotypes). (A) Appearance of p53. Presented is a Western blot analysis of p53 levels found in SaOS2 cell lysates at 24 h after transfection. The p53 proteins were detected with DO-1 and pAb1801 antibodies. Immunodetection of actin was used as a control. (B) Suppression of colony growth. SaOS2 cells were transfected using 1.5 μg of pCMV-Neo plasmids containing different *p53* alleles under the control of a cytomegalovirus promoter and subjected to drug selection. Colonies were counted after 12 to 14 days of selection. (C) Evaluation of apoptosis induction by mutant p53s. Twenty-four hours after transfection, cells were simultaneously stained with propidium iodide and acridine orange, as described in Materials and Methods. The relative percentages of apoptotic cells were determined based on nuclear morphology and stain pattern. The bar graphs show the averages of three independent experiments and include standard deviations (SD). WT, wild type.

Cell lines were isolated that expressed p53 at biologically meaningful levels. The levels were comparable to those induced by damage in wild-type cells that are capable of expressing endogenous p53 (see Fig. S1 in the supplemental material)

TABLE 1. Biological responses resulting from expression of wild-type and mutant p53s and the impact of UV or γ radiation

Biological response	Evaluation time	Expression	p53 examined (by group no.)									
			1		2		3	4		5		Null[b]
			WT	T123P (L1)[c]	S121F (L1)[c]	N288K (H2)[c]	T125R (L1)[c]	H178Y (L2)[c]	G279R (H2)[c]	G279E (H2)[c]	G245S (L3)[c]	
Growth suppression	3 wk	Transient/stable[d]	++++[a]	++++	+++++	+++++	++	+++	+++	+	+	−
Apoptosis	24 h	Transient/stable[e]	+++	+++	+++++	+++++	+(+)	+++	+++	−	−	−
Cell death	24 h	Stable[f]	+++	+++	++++	++++	+(+)	++	+++	−	−	−
UV clonal survival	3 wk	Stable[g]	++	++(+)	+	+	++++	+++	++++	+++++	+++++	+++++
UV-specific cell death	24 h	Stable[h]	++++	+++	++[j]	++(+)[j]	+(+)	++	+(+)	+	+	+
γ clonal survival	3 wk	Stable[i]	+++	+++	+	+	+	++	++	−	−	−
γ-specific cell death	24 h	Stable[h]	++	++	+++[j]	+++[j]	+	+	(+)	+	+	+

[a] Presented are qualitative summaries of responses relative to p53 null cells (SaOS2). Responses are derived from Figures 1, 2, and 3. The number of "+" marks corresponds to the level of effect, i.e., +++++ can indicate high level of cell death or high clonal survival. Parentheses indicate an intermediate value; i.e., +(+) means between one and two +'s.

[b] Results with SaOS2 cells which are deficient in p53.

[c] Structural domain (38).

[d] Colony suppression estimated from Figures 1B and 3B.

[e] Apoptosis estimated from Figures 1C (morphology), 2B (subG1-FACS), and 2C (annexin V).

[f] Cell death corresponds to apoptosis + necrosis as measured by annexin-V/7-AAD assay; see Figure 3A.

[g] Clonogenic assay from Figure 3C.

[h] UV- or γ-specific cell death; calculated by substracting the total dead cells 24 h after UV or γ treatment from the total dead cells resulting from expression of p53 only; see Figure 3A.

[i] Clonogenic assay from Figure 3D.

[j] Because of the high spontaneous level, this is a minimum estimate.

or in tumor cell lines that exhibit continuous mutant p53 expression (5). As shown in Fig. 2A, the selected clones for the wild-type p53 (DM wild type 10) and the mutants S121F (DM S121F-8), T125R (DM T125R-78), G279E (DM G279E-7), and G279R (DM G279R-76) did not produce p53 when cells were grown in the presence of dox. Removal of dox led to comparable levels of induction of the p53 protein, similar to levels detected in UV-irradiated $p53^+$ cell lines (RKO, HT1080, and U2OS cells) (see Fig. S1 in the supplemental material).

To address the effects of the various mutants on apoptosis, the presence of sub-G_1 cell populations at 24 h after dox removal was determined using flow cytometry (summarized in Fig. 2B). Apoptosis was also determined by the appearance of annexin V-stained cells (Fig. 2C). The patterns of apoptosis induction by the stably expressed mutants in relation to wt p53 were comparable to those found when the mutants were transiently expressed (summarized in Table 1).

Using FACS analysis and assessment of DNA content, the effects of the p53 mutants on cell cycle progression in the nonapoptotic cells were also determined (see Fig. S2 in the supplemental material). There was no effect on cell cycle profile for the mutants T125R, H178Y, G245S, G279R, and G279E or for cells that do not express p53. However, expression of the wild type and the T123P p53 mutant resulted in a decrease in the fraction of cells in S phase and an increase in G_1 cells. Cells expressing the p53 mutants S121F and N288K showed an even more prominent G_1 arrest and almost no S-phase cells, in addition to the high levels of apoptosis induced.

At 24 h after induction of p53, the presence of dead cells, which corresponds to the sum of apoptotic plus necrotic cells (see Materials and Methods), was assessed (Fig. 3A). The expression of the wild type, T123P, H178Y, and G279R resulted in similar frequencies of dead cells. The G245S and G279E proteins had little effect on cell death, while the T125R was intermediate between these and the wild-type protein.

p53 has broad biological effects on cellular responses to DNA damage. Its impact on survival following exposure to DNA-damaging agents reflects its influence on a variety of biological processes that include apoptosis, checkpoint arrest, and DNA repair. Surprisingly, although p53 is considered a "guardian of the genome," a lack of p53 can result in resistance to DNA-damaging agents, which may reflect opposing effects of these various processes (7, 31, 32, 59, 64). We therefore evaluated the consequences of mutational changes in the p53 regulatory network on sensitivities to UV and γ radiation measured as both clonal survival and damage-induced cell death, with the view that some alterations in the network might be beneficial and others might not.

To ensure that p53 would be present at the time of irradiation (see Fig. S3 in the supplemental material), the dox medium was changed to medium lacking dox at 6 h prior to exposure. Following UV (0, 5, or 10 J/m^2) or γ irradiation (0, 2, 4, or 8 Gy), cells were continued in the dox^- medium (p53 ON). In the absence of radiation, clonal survival varied from 20% for the S121F and N288K mutants to 30% for the wild type to ~100% for the transactivation-inactive mutants G245S and G279E (Fig. 3B), similar to growth suppression results obtained with transfectants (Table 1).

To determine the specific effects of the expressed p53s in response to UV, the clonal survival rates of irradiated cells were compared to those of unirradiated cells (Fig. 3C and Table 1). The presence of wt p53 greatly increased UV sensitivity compared to p53 null cells, while the mutant p53s had various impacts on UV tolerance. Cells expressing loss-of-function G245S and G279E mutant p53s, as well as untransfected SaOS2, were resistant to UV radiation. The presence of S121F and N288K mutant proteins increased sensitivity over that of wt p53, while cells expressing H178Y, G279R, or T125R were more resistant.

Loss of UV clonal survival due to the presence of wt or mutant p53s appears related to the ability of UV to induce cell

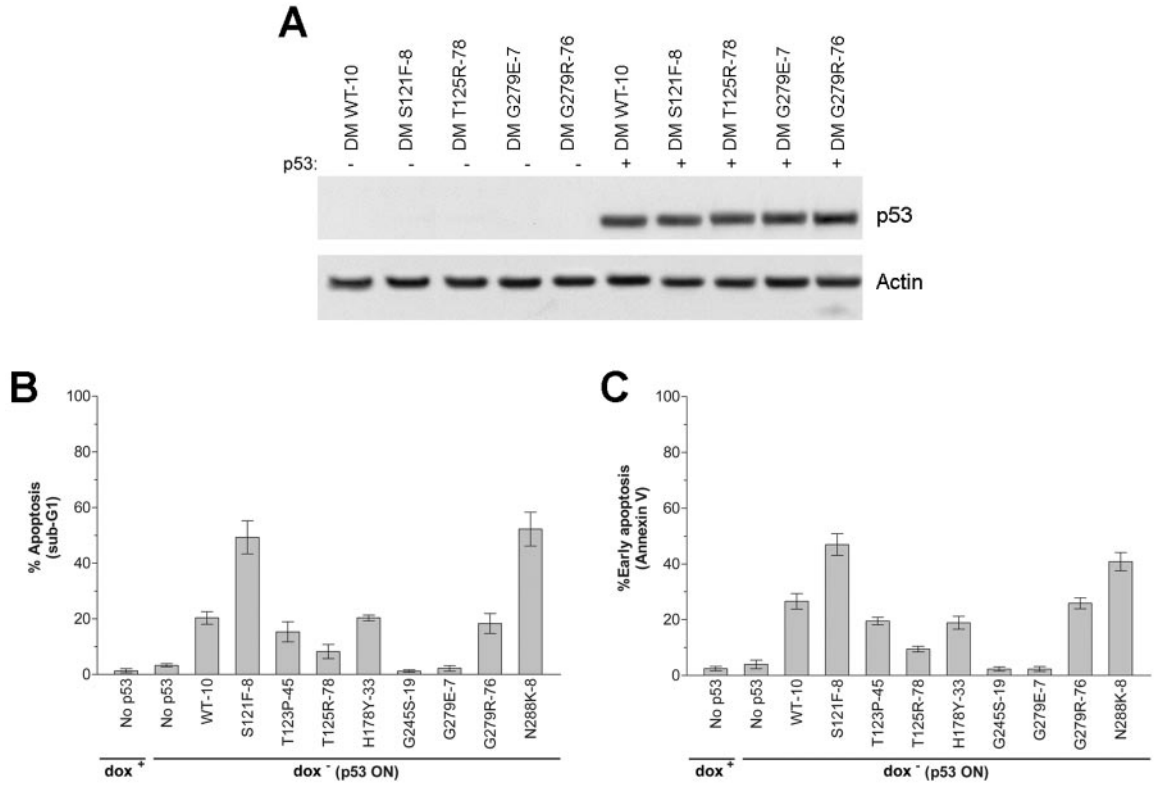


FIG. 2. Inducible regulated expression of p53 mutants results in different levels of apoptosis induction. (A) Western blot analysis of p53 induction. Stable SaOS2 cell lines with the different *p53* transgenes regulated by the Tet-Off promoter were cultured in medium containing dox. Lanes 1 to 5 correspond to $p53^-$. Lanes 6 to 10 correspond to cells growing for 24 h in medium lacking dox (i.e., p53 ON). Immunodetection of actin was used as an internal control. The numbers following the p53 designations refer to the cell lines. (B) Induction of apoptosis by expressed p53 mutants. SaOS2 cells were harvested 24 h after dox removal from the medium (i.e., p53 ON). The cells were stained with propidium iodide and subjected to flow cytometry. (C) Evaluation of apoptosis by annexin V staining and flow cytometry. SaOS2 inducible cell lines were harvested and counted 24 h after dox removal (p53 ON). Cells (1×10^5) from each sample were stained with PE-conjugated annexin V and then analyzed by FACS. Presented are the average percentages and SD from three independent experiments. WT, wild type.

death at 24 h via the p53 pathway (Fig. 3A and Table 1). Cells that were null for p53 or expressing the G279E or G245S proteins exhibited high clonal survival and little UV-induced cell death (i.e., "+++++" for survival and "+" for induced cell death in Table 1). Wild-type and mutant p53s that decreased clonal survival after UV also resulted in greater UV-induced cell death. The full impact of S121F and N288K proteins on UV-induced cell death could not be determined because of the high level of spontaneous cell death. Thus, wt p53 and some of the mutants reduce UV tolerance to various extents. The amount of early (24 h) UV-induced apoptosis/necrosis appears to be a good predictor of clonal survival.

The impact of expressed p53s in responses to γ radiation was quite different. Distinctly for UV, cells expressing wt p53 as well as T123P conveyed resistance, in terms of clonal survival, that was similar to that observed with $p53^+$ U2OS cells, while null cells or cells expressing either G279E or G245S were highly sensitive (Fig. 3D; summarized in Table 1). Expression of H178Y and G279R resulted in intermediate resistance. While their impact on UV clonal survival differed, cells expressing S121F, T125R, and N288K had similar effects, causing γ sensitivity to increase to nearly that of p53 null mutations.

Unlike results for UV, p53-dependent cell death 24 h after γ irradiation does not appear to be a good predictor of clonal survival. Cell death at 24 h after γ irradiation among mutants was also different from the UV responses (Fig. 3A). While cells expressing the wild type and T123P had high clonal survival after γ irradiation, they also exhibited substantial p53-dependent apoptosis/necrosis at 24 h. Although clonal survival was low, the expression of null mutants G279E and G245S did not result in high levels of cell death at 24 h. In spite of similar abilities of S121F, T125R, and N288K to decrease clonal survival, the T125R had much less of an effect on inducing cell death after γ irradiation.

***p53* mutations in the DBD result in at least five patterns of biological responses.** Overall, we found that the individual mutants can have very different effects on clonal survival and apoptosis/necrosis. As summarized in Tables 1 and 2, there were at least five distinguishable patterns of biological responses for the eight mutants examined when expressed at biologically relevant levels in human cells. The pattern 1 biological responses correspond to wt p53 and include T123P. The S121F and N288K mutants, comprising pattern 2 responses, have an impact that is stronger than the wild type. In pattern 3, observed with T125R expression, there are varied effects relative to the wild type depending on the end point examined, while in pattern 4 (H178 and G279R) there appear to be modest differences compared to the wild type. Pattern 5

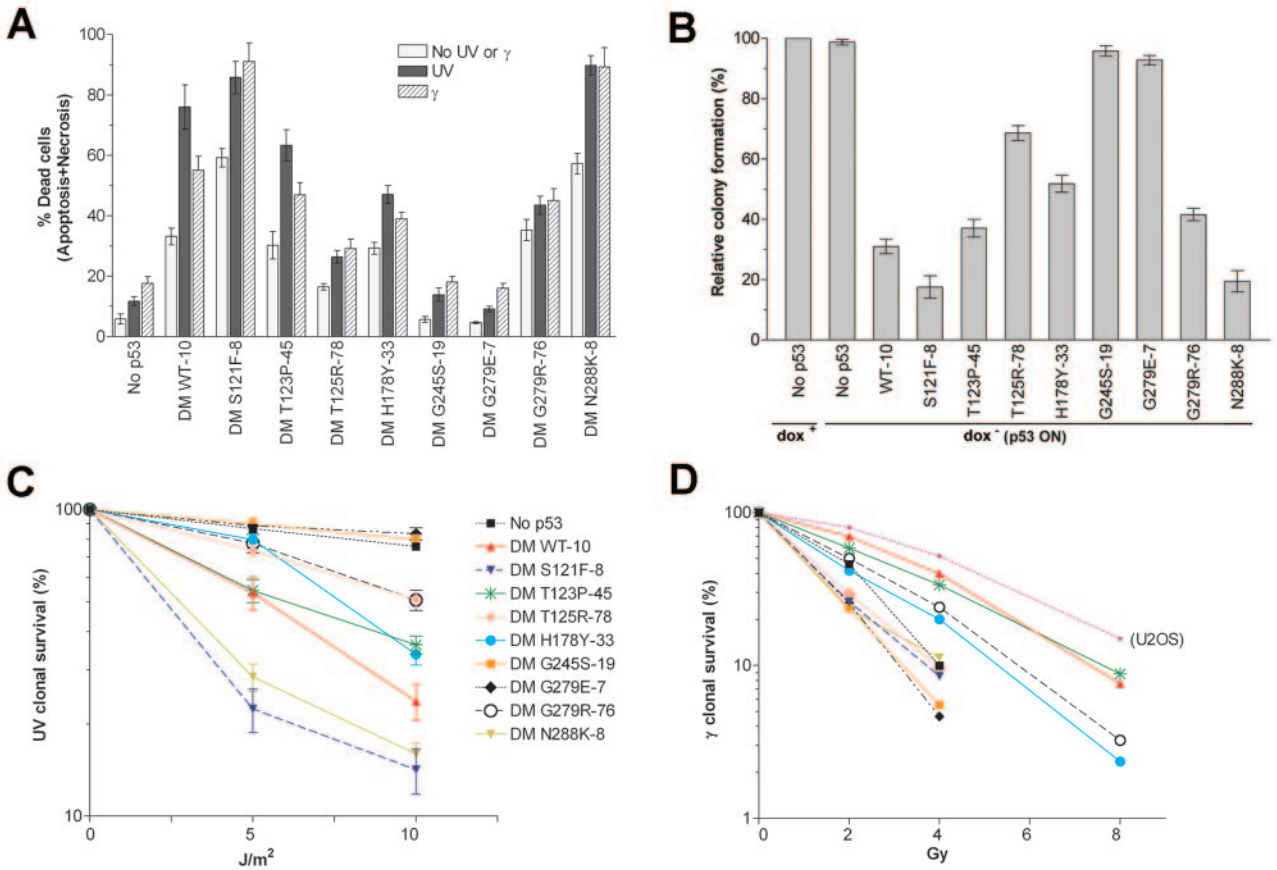
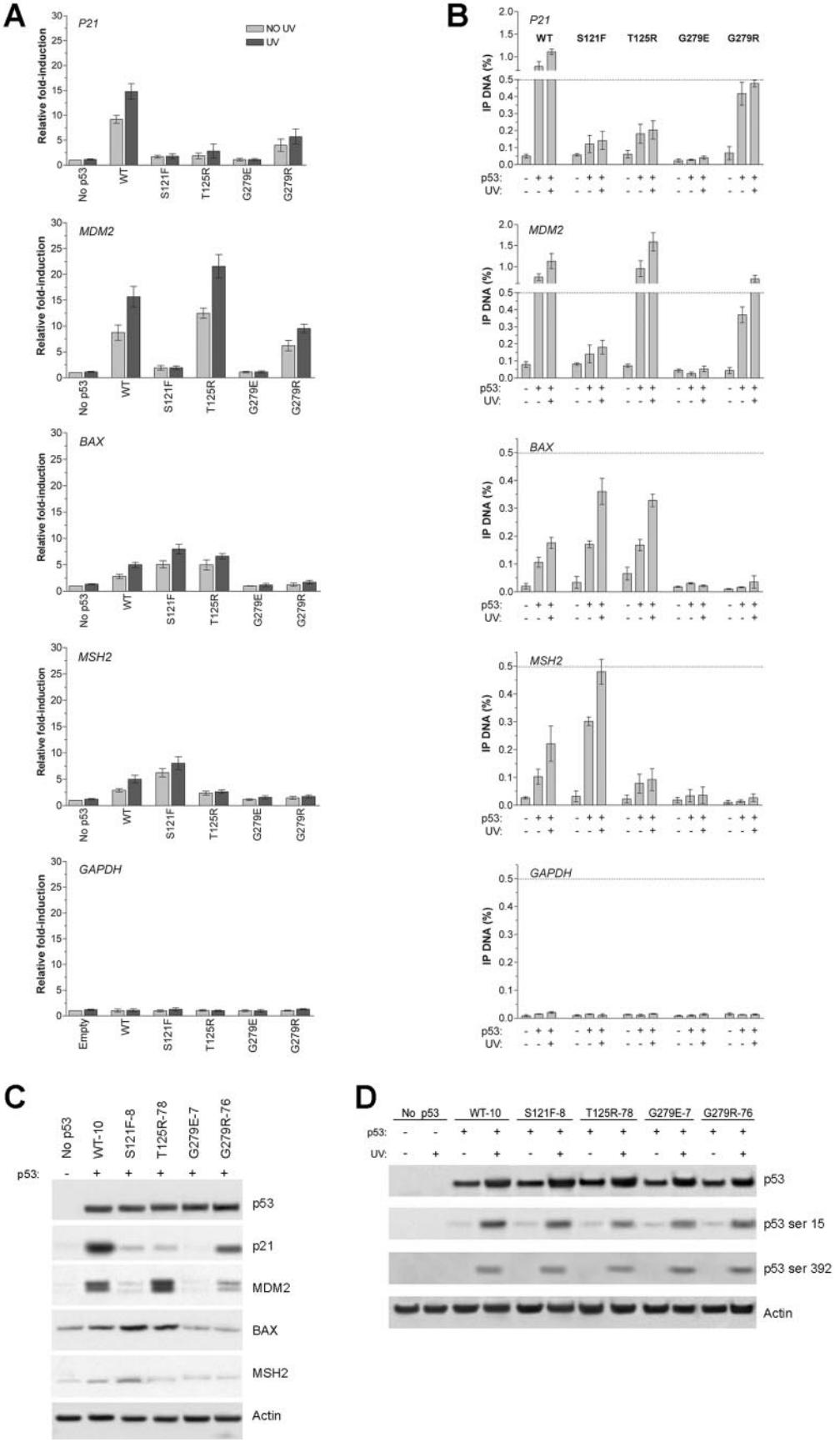


FIG. 3. Expression of p53 mutants affects resistance to UV and γ radiation. (A) Cell death. SaOS2 cells with p53 mutants under the Tet-inducible promoter were grown in the presence of dox (p53 OFF). Six hours before UV or γ irradiation, dox was removed (p53 ON). Cells were examined at 24 h after exposure to UV (10 J/m^2) or γ radiation (4 Gy) or no radiation. Total cell death is the sum of early and late apoptosis as well as necrosis quantified by flow cytometry analysis of 7-AAD- and annexin V-PE-stained cells. (B) Clonal survival. SaOS2 cells containing p53 mutants under the Tet-inducible promoter were grown in the presence of dox (p53 OFF) or in the absence of dox (p53 ON), and percent clonal survival was determined 21 days later. (C) Changes in sensitivity to UV resulting from expressed p53 mutants. (D) Changes in sensitivity to γ radiation resulting from expressed p53 mutants. Clonal survival of radiation-treated cells containing induced p53 was compared to p53 ON cells that were not irradiated. Presented are results and SD from three independent experiments. WT, wild type.

(G279E and G2345S) corresponds to complete deficiency in p53, similar to results with the null p53 cell line. One candidate from each group was examined for its ability to influence the activity of genes in the p53 regulatory network: wt, S121F, T125R, G279R, and G279E. Each of the mutations has been identified in human tumors.

Expressed p53 mutants result in a variety of p53 target gene activities. Given the differences in biological responses, we addressed whether they are specifically attributable to altered capabilities for transactivation. Three facets of activity of p53 target genes were assessed following the induction of wt p53 and mutant S121F, T125R, G279R, and G279E proteins: transcription, as determined by real time PCR; chromosomal occupancy by p53; and induction of proteins encoded by the target genes. While a direct relationship might be assumed, there are many factors, such as coactivators, stress, and the

FIG. 4. Transactivation patterns resulting from expression of p53 mutants correlate with p53 occupancies at endogenous promoters and protein levels from target genes. SaOS2 cells containing stable Tet-Off-inducible p53s were induced for p53 (dox off) and 6 h later treated or not treated with 10 J/m^2 UV. The cells were continued in the p53-inducing medium and analyzed at 24 h. Presented are the mean values and SD from three independent experiments. (A) Differential induction of p53 target genes. Transactivation activities were analyzed by real-time PCR. The levels of mRNA expression are presented as induction relative to cells lacking p53. (B) Expression of p53 results in differential promoter occupancies by p53 mutants. ChIP analysis was done 24 h posttreatment (+ or − UV) using a p53-specific antibody (see Materials and Methods). The bar graphs represent the percentages of immunoprecipitated DNA with respect to input samples. For gene expression and ChIP assays, *GAPDH* was used as negative control. (C) Inducible expression of p53 mutants changes the protein expression spectra of several p53 target genes. Cell lysates were subjected to Western blot analysis. (D) Comparable p53 stabilization and posttranslational modifications in wild-type and p53-mutant proteins after UV. At 24 h post-UV treatment, phosphorylation of p53 at Ser 15 and Ser 392 was analyzed by Western blot using specific monoclonal antibodies. Actin was the internal loading control. IP, immunoprecipitated; WT, wild type.


A
P21
NO UV
UV
Relative fold-induction
No p53
WT
S121F
T125R
G279E
G279R
MDM2
BAX
MSH2
GAPDH
Empty
B
P21
WT
S121F
T125R
G279E
G279R
IP DNA (%)
p53:
UV:
MDM2
BAX
MSH2
GAPDH
C
No p53
WT-10
S121F-8
T125R-78
G279E-7
G279R-76
p53:
p53
p21
MDM2
BAX
MSH2
Actin
D
No p53
WT-10
S121F-8
T125R-78
G279E-7
G279R-76
p53:
UV:
p53
p53 ser 15
p53 ser 392
Actin

mutants themselves, that might influence the relationship. Gene activity was determined for the following genes: *P21*, associated with S-phase cell cycle arrest; *MDM2*, which prevents accumulation of p53; *BAX*, associated with apoptosis; and *MSH2*, the DNA mismatch repair gene associated with damage-induced apoptosis. *MSH2* was also chosen because p53 control of the endogenous gene had not previously been described. The wt and p53 mutants were expressed from the stably integrated, dox-regulatable cassettes described above, and gene activity measurements were determined 24 h after induction.

Transactivation patterns differ between the p53 mutants. The spectra and levels of genes transactivated by each of the mutant p53s are distinct from the wild type, as described in Fig. 4 (for endogenous p53 target genes) and Fig. 5 (transfected plasmids with p53 target gene REs placed upstream of a luciferase reporter) and summarized in Table 2. The S121F, T125R, and G279R mutants clearly retain p53 function based on their abilities to transactivate target genes. The S121F (supertrans in yeast) was greatly reduced for *P21* and *MDM2* (similar to results of Kaeser and Iggo [25]) but supertrans for *BAX* and *MSH2*, whereas T125R was supertrans for *MDM2* and deficient for *MSH2* and *P21*. The G279R was only able to transactivate the *P21*and *MDM2* genes (G279R and T125R were change-in-spectrum in yeast with enhanced activity on the *MDM2* RE). It is interesting that single amino acid changes G → R or G → E at codon 279 could change the pattern of transactivation dramatically. Thus, the differences in biological response patterns reflect, in some way, specific differences in spectra and levels of gene induction. The results with the expressed transgene were comparable to those with transfected p53 plus RE::luciferase reporters for *P21*, *MDM2* and *MSH2* REs (Fig. 5) (23).

The transactivation activities of S121F, T125R, and G279E towards additional endogenous target genes involved in apoptosis, DNA repair, cell cycle, and stress pathways were also examined using transfected p53 (see PCR results in Fig. S4A in the supplemental material). The cell cycle and stress genes *Cyclin-G*, *GADD45*, *MDM2*, and *P21* were all highly induced (approximately fivefold) by wt p53. The repair genes *MSH2* and $P48^{DDB2}$ were induced three- to fivefold, but there was little induction of *XPC*. Induction of the apoptotic genes *AIP1*, *APAF*, *BAX*, *PIG3*, and *PUMA* varied from two- to fivefold. Transient transfection by the supertrans S121F resulted in increased transactivation relative to the wild type for the apoptotic genes *AIP1*, *APAF*, *PIG3*, and *PUMA*, but there was a change in spectrum for the cell cycle/stress genes and the DNA repair genes. The expression profile was very different for the T125R allele which only induced *MDM2*, *BAX*, and *PIG3* transcription and not the repair genes *MSH2*, $P48^{DDB2}$, or *GADD45*. The DNA-binding deficient mutant G279E had no impact on expression from any of the genes. The differential effects on the expression of DNA repair genes suggest differences in repair that might be addressable with these mutants.

Transactivation patterns correlate with p53 occupancies at endogenous promoters and induced target protein levels. While transactivation requires direct interaction of p53 with REs, many factors can contribute to the final levels of transcription. We investigated the relationship between occupancy and extent of transactivation from the endogenous target genes, as well as the consequences of DNA damage on these

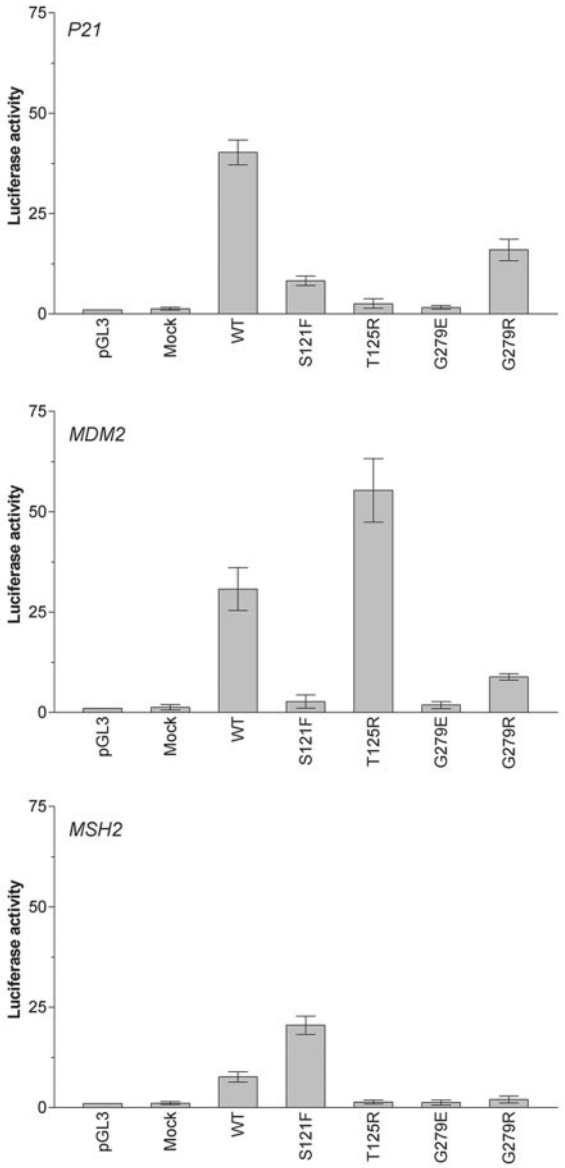


FIG. 5. Analysis of transactivation by the wild type and various p53 mutants at p53-responsive promoters. The *P21*, *MDM2-P2*, and *MHS2* promoters were tested. Plasmids containing luciferase reporter constructs were cotransfected together with p53 expression plasmids and the control vector pRL-SV40 into SaOS2 cells, as described in Materials and Methods. Cell lysates were prepared 48 h after transfection and luciferase activity was measured. Results were normalized using the *Renilla* constitutive luciferase activity. The relative means and the SD for at least three independent experiments are presented. WT, wild type.

two features of gene expression. Occupancy by the various wild-type and mutant p53s at the *P21*, *MDM2*, *BAX*, and *MSH2* response elements was determined 24 h after removing dox using ChIP. The results are presented in Fig. 4 and summarized in Table 2 as target gene activity. There was high occupancy by wild-type p53 at promoters of the strongly expressed *P21* and *MDM2* genes (0.8%) and low occupancy for the weakly transactivated genes *BAX* and *MSH2* (0.1%). The patterns of occupancy by the mutants were comparable to those

TABLE 2. Target gene activity resulting from expression of wild-type and mutant p53s and the impact of UV or γ radiation

Condition	Gene	Target gene activity[a]					
		WT	S121F	T125R	G279R	G279E	Null
NO UV[b]	*P21*	+++	+	+	++	−	−
	MDM2	+++	+	++++	++	−	−
	BAX	++	+++	+++	−	−	−
	MSH2	++	+++	−	−	−	−
+UV[c]	*P21*	++++	+	+	+++	−	−
	MDM2	++++	+	+++++	+++	−	−
	BAX	+++	++++	++++	−	−	−
	MSH2	+++	++++	−	−	−	−

[a] Presented are qualitative summaries of responses relative to p53 null cells (SaOS2). Responses are derived from Fig. 4. The number of "+" marks corresponds to the level of effect, i.e., +++++ indicates the highest level of induction.
[b] Corresponds to gene expression/promoter occupancy/protein expression (see Fig. 4).
[c] Corresponds to gene expression/promoter occupancy (see Fig. 4).

for transactivation, suggesting that occupancy is a good predictor of transcription for the regulatable wild-type and mutant p53s. The S121F mutant had markedly increased occupancy for *MSH2* (0.3%), somewhat higher than the wild type for *BAX*, and much lower for *P21* and *MDM2* promoters. For cells expressing the T125R allele, only the *MDM2* promoter had a large increase in occupancy (0.9%). No occupancy was observed at the *BAX* and *MSH2* promoters for the G279R mutant, but there was substantial occupancy at the *P21* (0.5%) and *MDM2* (0.4%) promoters. There was no significant binding by G279E protein at any of the promoters. Nonspecific precipitation of *GAPDH* DNA, used as a negative control, was minimal (0.02%) for wild-type p53 and the altered p53 alleles. Thus, differences in transactivation capacity towards the various REs are best explained as being due to differences in the ability of the proteins to bind rather than in the capability for transcription once bound.

As might be expected, the appearance of the P21, MDM2, BAX, and MSH2 proteins was directly related to transcription of the endogenous genes (Fig. 4C) (also found for transfected p53 mutants, as shown in Fig. S4B in the supplemental material). For example, whereas wild-type p53 increased the levels of the four proteins, the levels of MSH2 and BAX were low although somewhat higher than in the p53 null SaOS2 cells. For S121F, the levels of BAX and MSH2 were higher than the wild type but much lower for p21 and MDM2, while the level of MDM2 was higher than the wild type with the T125R mutant. Thus, the different patterns of biological responses relate to changes in levels of proteins induced by the various mutants.

Effects of p53 and UV on gene activity. To address the impact of stress due to UV irradiation on gene activity, occupancy and transactivation of target genes were examined. At 6 h following dox removal, the p53-induced cells were exposed to 10 J/m^2. Densitometric analysis (data not shown) of the blots showed that UV exposure led to a threefold increase in p53 levels in all cell lines 24 h after the treatment (Fig. 4D). The various p53s did not differ in their ability to undergo UV-induced phosphorylation at serines 15 and 392 (Fig. 4D), suggesting that the wild-type and mutant proteins were equally capable of being stabilized in response to UV.

The UV-induced changes in the p53 proteins had the general effect of amplifying the increases in transactivation observed with expression of wild-type and mutant p53s in unirradiated cells (Fig. 4A and Table 2). However, the low and modest levels of *P21* transactivation by S121F, T125R, and G279R were not increased by UV exposure (similar to *MDM2* transactivation by S121F). Genes that did not respond to the mutant p53s remained unresponsive following UV irradiation. Promoter occupancies by the various p53s directly reflected the UV-induced changes in transactivation (Fig. 4B) at each RE. Thus, the increases in transactivation resulting from UV exposure are likely due simply to increases in the amounts of p53 proteins (Fig. 4D) as well as occupancies. It is interesting that while the amounts of p53 proteins could be increased by UV, in some cases the occupancies and associated transactivation by some of the mutants at particular promoters did not change. This may indicate that other factors affect binding by the mutant proteins at particular promoters following UV exposure.

DISCUSSION

Cancer is the culmination of many genetic and gene expression changes. The localized evolution enables tumorigenic cells to compete more effectively in restricted environments. Our studies with the master regulatory gene *p53* provide insights into how single tumor-associated mutations can differentially change a vast expression network and thereby yield a variety of cellular phenotypes.

Based on results with expressed human mutant p53s in a yeast system (42) we had hypothesized that master regulators such as p53 could also be master genes of diversity. This hypothesis suggested that mutations could change the spectra of genes targeted by p53, as well as transactivation intensities. Ultimately, altered biological responses could result. To test this hypothesis, with a view toward understanding how various mutants might contribute to cancer as a biological end point, we examined mutants that had been divided into three categories based on transactivation capacities when expressed in yeast: supertrans, change of spectrum, and inactive. Although the p53 mutants selected for this study are infrequently associated with cancers, change-of-spectrum mutants provide a good opportunity to address specific gene transactivation functions that may be relevant to biological end points.

Overview of impact of wt and p53 mutants on biological responses and transactivation. The impacts of the mutants on biological activities affected by p53 in human cells were exam-

ined at biologically relevant levels of protein expression. As summarized in Tables 1 and 2, the eight DBD mutants tested had markedly different biological consequences, resulting in five patterns for the six end points examined: growth suppression, spontaneous apoptosis, and UV and γ effects on clonal survival and on cell death. Since these are only a subset of possible biological stress responses, sampling a larger set of mutants and more biological end points should lead to an even greater number of patterns and, therefore, phenotypes. Using a representative from each pattern and a subset of genes in the p53 network, we established that the patterns of biological responses reflect differences in the activity of p53-responsive genes, as summarized for four mutants in Table 2. Additional information for more target gene activities comes from transient expression, described in Fig. S4 in the supplemental material. While over 50 genes are targeted by p53, expression changes in the target genes examined provide insights into how the altered spectra of gene activities might relate to the observed patterns of biological responses.

As expected, expression of wt p53 in the p53 null cell line causes growth suppression and apoptosis which are likely related to the induction of *P21*, *BAX* (Table 2), and other apoptotic genes (see Fig. S4 in the supplemental material). It also sensitizes cells to UV, probably through increased apoptosis. Interestingly, there is a reduction in survival even though p53 was able to induce genes involved with repair of UV damage ($P48^{DDB2}$ and *XPC*) (also found for other p53 mutants). Unlike the case for UV, p53 provides protection against γ exposure. For both UV and γ radiation the impact of p53 on UV sensitivity or γ resistance is related to its sequence-specific transactivation capabilities (compare the wt versus G279E and null).

While the reduction in UV resistance resulting from expression of mutant as well as wt p53s is related to induction of apoptosis, the role for apoptosis in γ radiation survival responses is not clear based on results with the various p53s. Cells expressing some mutants, including the null, are highly sensitive in terms of clonal survival but exhibit low levels of cell death (apoptosis plus necrosis). On the contrary, cells expressing the wild type and T123P show high levels of clonal survival but also yield high levels of cell death. Furthermore, survival is similar for cells with expressed S121F and T125R, yet S121F causes much more γ radiation-induced apoptosis. Thus, we conclude that whether γ irradiation induced early cell death is not a predictor of sensitivity. Other factors, such as the types of damage induced by UV and γ radiation, the activation of repair pathways, and expression of other genes not examined in this study, may also contribute to the differences in sensitivity resulting from the expression of the p53 change-of-spectrum mutants.

The mutants also provide the opportunity to address the role of specific p53-targeted genes in various responses. Overall, the results with the various mutants demonstrate that change-of-spectrum mutants provide opportunities to separate out a general role for the p53 protein in biological responses (i.e., interactions with other factors) versus its role in transcriptional control of specific genes.

Altered responses due to S121F mutant. The S121F mutant was identified as supertrans in yeast, but change-of-spectrum in human cells. In SaOS2 cells, it generally had a more dramatic effect on biological end points than wild type p53, including high levels of spontaneous apoptosis as well as low clonal survival following exposure to UV and γ radiation. These responses were consistent with the reduced level of *P21* and the up-regulation of many of the endogenous p53 target genes in the apoptosis pathway (Table 2) (see Fig. S4 in the supplemental material) including *MSH2* (see below). Our results are consistent with the higher apoptosis reported in several other human cell lines (26, 45) through the combination of reduced *MDM2* induction and increased or unbalanced expression of other apoptosis-inducing p53 target genes (1, 45). We note that, unlike results for the various endogenous genes in the present study, Kakudo et al. (26) found that the S121F mutant generally resulted in reduced transactivation among the RE reporters examined in a transient transfection system.

The T125R change-of-spectrum mutant. A very different pattern of biological responses and spectrum of target gene activity relative to the wt and S121F were observed for the T125R mutant (Tables 1 and 2). The p53-specific biological responses were generally reduced from those in the wild type. The T125R protein caused less spontaneous growth suppression and apoptosis and it was similar to the null in that there was little UV-associated cell death or loss of clonal survival and the cells remained γ sensitive. The increased *BAX* and *PIG3* expression (Table 2) (see Fig. S4 in the supplemental material) argues against these genes having specific roles in spontaneous or DNA damage-induced apoptosis, at least when controlled by T125R.

The possibility that this mutant affects the expression of other genes that antagonize or attenuate apoptosis cannot be excluded. For example, Minsky and Oren (35) showed that enhanced MDM2 protein expression (a feature of T125R) (Table 2) causes silencing of some p53-targeted promoters through MDM2-mediated histone monoubiquitylation and other chromatin modifications.

G279E loss-of-function versus G279R change-of-spectrum mutants. The two amino acid changes at position 279, glycine to the negatively charged glutamic acid or the positively charged arginine, had markedly different effects on p53-associated end points. The mutation G279E was essentially null, although its presence did cause a slight increase in spontaneous apoptosis. On the other hand, the G279R mutation, which exhibited change-of-spectrum gene activities, retained some p53-associated biological properties including near-wild-type levels of spontaneous apoptosis in spite of the absence of *BAX* induction and intermediate resistance to γ radiation.

Results with T125R and G279R provide an opportunity to address a possible role for *MSH2* in apoptosis and lethality. In addition to being essential in mismatch repair, *MSH2* links DNA damage to apoptosis and cell cycle regulation (12, 19, 21, 54, 62). We established that p53 can induce expression of the endogenous *MSH2* gene, supporting previous observations with a transfected *MSH2* reporter (47). Expression of the wt, S121F, and G279R, but not T125R, resulted in substantial spontaneous apoptosis (Fig. 3A and Table 1). Yet neither G279R nor T125R induced *MSH2.* The induction of apoptosis in UV- or γ-irradiated cells containing wt p53 was not found for G279R- or T125R-expressing cells. Possibly MSH2 participation in the apoptotic pathway depends on the presence of DNA damage.

Transactivation and occupancy by p53. The present experiments also address the relationship between the level of p53 and

stress-induced transactivation. p53 was expressed constitutively from a regulatable promoter at levels corresponding to those found in $p53^+/p53^+$ cells that are stressed (see Fig. S1 in the supplemental material) or in tumors that have mutant p53 proteins (14, 25). In our system, the transcriptional responses to the induced p53 at the four target genes examined were directly proportional to the occupancy. UV stress and subsequent modification of p53 did not markedly change the level of occupancy, arguing against a latency model for p53-induced gene activation, in agreement with other reports (25). Increases in nuclear p53 protein, rather than allosteric modifications, are associated with transcriptional activation (4, 33). The additional occupancy following UV may be due to increased synthesis of p53, stabilization, and/or phosphorylation (Fig. 4D).

Espinosa et al. (14) demonstrated with endogenously expressed p53 in $p53^+/p53^+$ cells that stresses can differentially alter the expression of p53 target genes. They showed that while there is a requirement for p53, recruitment of additional transcriptional factors and chromatin modifications play important roles in the kinetics of target gene induction following stress. Cofactors such as ASPP1 and even other p53 family members p63/p73 can selectively enhance the p53 apoptotic activity by facilitating its binding to proapoptotic promoters at least in some cell types (15, 46, 50). It should be noted that for unstressed $p53^+/p53^+$cells, p53 is bound to some of its target promoters (3, 13, 14, 53), and the eventual p53 transactivation following stress may not be directly related to occupancy (25, 53). In our case, there was no dramatic increase in transcription resulting from the UV stress other than a change that could be ascribed to the modest increase in p53 protein. For the two weaker promoters (*BAX* and *MSH2*) there was no increase in occupancy nor associated transcription. The difference in results for occupancy and transcription between ours and those of Espinosa et al. (14) could rest in the relatively high level of expressed p53 in our system and/or the establishment of transactivation prior to stress. In our system the transcriptional complex may already be in place (i.e., primed) at the time of exposure.

Conclusions and implications. Since many p53-associated biological properties are the result of transactivation of targeted genes by p53, changes in transactivation properties might be expected to result in altered biological properties. We have linked biological outcomes with changes in the ability of mutant and wt p53s expressed at biologically relevant levels to directly regulate p53 target genes. Recent experiments of Kakudo et al. (26) examining apoptosis 3 days after transfection of mutants into SaOS2 cells suggest that p53 mutants can also increase spontaneous apoptosis through mechanisms that do not involve dramatic changes in the transactivation patterns, based on results with six REs that include three from apoptotic genes.

In terms of connecting biological outcomes with *p53* mutations, our study is not exhaustive, yet it provides a framework for understanding various tumor-associated mutations and effects on tumor status and therapeutic responsiveness. Possibly, approaches to treatment of tumors with p53 defects will depend in part upon the consequences of individual mutations.

How the single codons affect binding and transactivation of different promoters will require further physical investigations involving nuclear magnetic resonance and x-ray crystallographic techniques. Unfortunately, there is little information that addresses p53 multimer interactions with its various target sequences. Mutants and response elements such as those described here could help guide those studies.

Overall, these results support the hypothesis that a single master regulatory gene such as *p53* can be a master gene of diversity by virtue of various individual mutations giving rise to different phenotypes. As described previously using a piano analogy (42), functional mutations in a master regulator (the *p53* hand) that change both the spectrum and intensity of downstream gene expression responses (the keys) can result in diversity (new or altered chords), which could be an important factor in governing environmental responses and the potential for disease (the "sounds," i.e., phenotypes).

Functional single-nucleotide polymorphisms in p53 response elements within the human population (as we recently reported) (34, 55) further amplify the diversity in responses that can arise from changes in p53 transactivation and could greatly expand the disease consequences of specific *p53* mutations. Finally, our findings have important implications for other master regulatory genes that recognize REs related to common consensus sequences or regulate indirectly large transcriptional networks. Along this line, parallel evolution of the bony armor of stickleback fish has been attributed to changes in the *Ectodysplasin* gene, which is included in the tumor necrosis family of secreted signaling molecules (9).

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